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Cytokines in human renal interstitial fibrosis. II. Intrinsic interleukin (IL)-1 synthesis and IL-1-dependent production of IL-6 and IL-8 by cultured kidney fibroblasts

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Cytokines in human renal interstitial fibrosis. II. Intrinsic Interleukin (IL)-1 synthesis and IL-1-dependent production of IL-6 and IL-8 by cultured kidney fibroblasts. We compared cytokine production from transformed human fibroblast cell lines derived from either a kidney with interstitial fibrosis or a normal kidney to that from primary human foreskin fibroblasts. Fibrosis-derived as well as normal renal fibroblasts, but not skin fibroblasts, spontaneously produced the chemokine, IL-8, and the growth promoting cytokine, IL-6. Spontaneous IL-8 and IL-6 synthesis by renal fibroblasts was dependent on the intrinsic release of IL-1, since blocking IL-1 receptors with IL-1 receptor antagonist (IL-1Ra) partially inhibited the constitutive production of these cytokines. Both kidney cell lines had detectable mRNA and protein for IL-1α and IL-1β. Renal and skin fibroblasts stimulated by picomolar concentrations of exogenous IL-1 or TNF-α produced large amounts of IL-6 and IL-8, whereas nanomolar concentrations of basic fibroblast growth factor did not. Fibrosis-derived cells expressed less high affinity IL-1 receptors (600 receptors/cell; K_D = 0.6 pM) compared to normal renal fibroblasts (1000 receptors/cell). However, fibrosis-derived renal fibroblasts produce three- to fourfold more IL-8 and IL-6 in response to picomolar concentrations of IL-1β compared to cells derived from a normal kidney. As this enhanced production is not due to increased numbers of IL-1 receptors, we speculate that post-receptor responsiveness to either endogenous or exogenous IL-1 is greater in fibrosis-derived renal fibroblasts than in cells from normal kidneys.

There is increasing evidence that cytokines and growth factors affect the onset as well as progression of renal inflammation [1–3]. Renal cells including glomerular mesangial cells and epithelial cells are activated by the pro-inflammatory cytokines interleukin-1 (IL-1) [4] and tumor necrosis factor (TNF) [5] to express adhesion molecules [6, 7], and to produce chemokines such as IL-8 [8–10] and monocyte chemotactic protein-1 (MCP-1) [11]. Furthermore, proliferation of mesangial as well as epithelial cells is enhanced by growth factors such as platelet-derived growth factor (PDGF) [12, 13], IL-6 [14], basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) [15]. In contrast, mesangial cell growth is inhibited by transforming growth factor (TGF)-β [16]. In addition to their responsiveness to IL-1 and TNF, glomerular mesangial cells synthesize these cytokines [4, 5].

The pathogenic processes whereby renal function is lost in the course of chronic glomerulonephritis (GN) is not fully understood. In addition to the progression of the primary inflammatory process in the glomerulus, the accompanying renal interstitial inflammation is associated with the decline in kidney function [17]. In fact, morphometric studies revealed that the decrease in creatinine clearance in chronic GN correlates with the degree of renal interstitial volume expansion [18–20]. Furthermore, the number of infiltrating lymphocytes and macrophages in the interstitium correlates well with the decline in kidney function [21, 22].

These data suggest that inflammatory processes in the renal interstitium play an important role in the progression of primarily glomerular disease to one of renal fibrosis and loss of kidney function [23]. Tubular interstitial fibroblasts participate in this process as demonstrated by studies using cultured cells from mice or rabbits [24, 25]. Human fibroblasts derived from a kidney with GN and interstitial fibrosis exhibit an increased rate of spontaneous proliferation when cultured *in vitro* [26]. This increased proliferation depends, in part, on the intrinsic production of IL-1 (see preceeding paper in this issue).

In the present study we investigated whether human renal fibroblasts produce the chemokine IL-8 as well as the growth-promoting cytokine IL-6. We studied whether IL-1β, TNF-α, and bFGF induced IL-6 and IL-8 production in two immortalized human renal fibroblast cell lines, one established from a kidney biopsy with GN and interstitial fibrosis and a control cell line derived from a normal kidney. Primary human foreskin fibroblasts were tested in comparison. Cells were studied for IL-1-dependent cytokine production using the IL-1 receptor antagonist (IL-1Ra) which blocks IL-1 receptors without agonist activity [27].

Methods

Cytokines

Recombinant human IL-1β (10^8 U/mg) was a gift of Dr. Aldo Tagliabue (Sclavo, Siena, Italy). Recombinant human IL-1 receptor antagonist (IL-1Ra) was a gift of Dr. Daniel Tracey (Upjohn Co., Kalamazoo, MI, USA) [27]. Recombinant human (TNF-α, 5 $\times$ 10^7 U/mg) was obtained from Genentech Inc. (South San

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Francisco, CA, USA). Recombinant human bFGF was a gift from Synergen Inc. (Boulder, CO, USA). Recombinant human IL-1α (2×10^8 U/mg) was a gift from Peter Lomedico (Hoffmann-LaRoche, Nutley, NJ, USA). For binding assays, 5 μg of human recombinant IL-1α were labeled with 0.5 mCi of ^{125}Iodine using the chloramine T method to a specific activity of 40 μCi/μg [28].

Cell cultures

Human renal interstitial fibroblasts were obtained from the medullary part of a kidney biopsy with histologically proven GN and interstitial fibrosis [26] or from the medulla of a normal human donor kidney. Several one mm^3 pieces of renal tissue from the medulla were added to petri dishes covered with tissue culture medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma), 10 mM Hepes (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY, USA), and 20% (vol/vol) fetal calf serum (FCS; HyClone Inc., Logan, UT, USA). Cells growing from the tissue specimens were identified as epithelial cells and fibroblasts by morphologic criteria and immunophenotyping [29, 30]. The mixed cultures were treated with trypsin (0.05%)/EDTA (0.1%) (Gibco) and subcultured in limiting dilutions in DMEM 20% FCS. Single cell type colonies were surrounded by siliconized glass rings and trypsinized. Fibroblast clones as identified by morphologic criteria were subcultured in DMEM, 10% FCS.

Clones of these early passage human renal fibroblasts were immortalized by transfection with the SV40 plasmid pSV3gpt [31]. Stable cell lines of these SV40 immortalized fibroblasts were obtained by repeated subcloning of transfected cells [32]. The renal fibroblast cell lines are comparable to the parental non-transformed interstitial fibroblasts as indicated by similar morphology, an unchanged expression pattern of collagens, fibronectin, laminin and vimentin as well as the negative immunostaining for cytokeratin, desmin and factor VIII in both cell lines [30, 32]. The two renal fibroblast cell lines (fibrosis-derived renal fibroblasts and normal renal fibroblasts) were maintained in RPMI 1640 (Sigma) containing L-glutamine, Hepes, antibiotics and 10% (vol/vol) Serum Plus® (JRH Biosciences, Lenexa, KS, USA). Renal fibroblasts were studied during passages 15 to 30.

Human skin fibroblasts were prepared from an infant foreskin and grown to confluence in RPMI containing 10% Serum Plus in 75 cm^2 tissue culture flasks (Costar, Cambridge, MA, USA). Skin fibroblasts were subcultured in RPMI 1640 containing 10% Serum Plus and studied during passages 5 to 20.

Fibroblast incubations

Fibroblasts were washed in PBS, trypsinized (0.05% trypsin, 0.1% EDTA) for two minutes at 37°C, diluted in RPMI containing 10% Serum Plus to inactivate the trypsin and centrifuged for 10 minutes at 250 $\times$ g. Cell pellets were resuspended in culture medium (RPMI containing 10% Serum Plus) and quadruplicate samples of 200 μl aliquotes of cell suspension were added to 96-well flat bottom microtiter plates (Costar). The cell number added to each well was approximately 5,000 skin fibroblasts and approximately 25,000 renal fibroblasts. After incubation for 24 hours, fibroblast supernatants were replaced by 200 μl of fresh culture medium alone (control), or by culture medium containing various concentrations of IL-1β, TNF-α, or bFGF. Fibroblasts were then incubated for additional 24 hours and supernatants were removed for the measurement of IL-6 and IL-8 by radioimmunoassay.

In order to express the amounts of IL-8 and IL-6 produced as a function of cell number, cell proliferation was determined in each experiment by means of the XTT assay. In this proliferation assay, the turnover of the tetrazolium salt XTT, 3,3′-[1-[(phenylamino) carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)-benzenesulfonic acid, hydrate, sodium salt (Polyscience, Inc. Warrington, PA, USA) into its water soluble formazan product was measured as an indicator of fibroblast proliferation [33]. There is a strong linear correlation between results of the XTT assay and number of cells counted in parallel fibroblast cultures after trypsinization (details are in the preceeding paper, this issue). Therefore, results of the XTT assay can be used to adjust for relative changes in cell numbers in stimulated fibroblast cultures compared to unstimulated controls. Results of the XTT assay were plotted against cell counts to obtain standard curves for each of the three fibroblast cell lines. Separate standard curves for each cell line were necessary because the XTT turnover per cell was approximately fivefold higher in skin fibroblasts than in both renal fibroblast cell lines (see preceeding paper). The standard curves were used to express the amount of cytokine produced per 5,000 skin fibroblasts or 25,000 renal fibroblasts, respectively.

In additional experiments, renal fibroblasts were incubated in triplicate samples in 24 well plates in order to determine spontaneous as well as IL-1-induced IL-1 synthesis. Total IL-1α and IL-1β were measured in cell lysates of duplicate samples after three freeze-thaw cycles by radioimmunoassay. Fibroblasts in the corresponding third well of each sample were trypsinized and counted in a Neubauer hemacytometer. The obtained cell counts were then used to correct the measured amounts of IL-1 for cell numbers.

Radioimmunoassay for IL-1α, IL-1β, IL-6 and IL-8

Cytokines were measured in fibroblast culture supernatants or in total cell lysates after three freeze-thaw cycles by specific radioimmunoassays (RIA). Each RIA was performed as described previously [28, 34–36]. The Bolton-Hunter method [37] was used to label IL-1β. Bolton-Hunter labeled ^{125}I-IL-6 was purchased from New England Nuclear (Billerica, MA, USA). The concentration of cytokine resulting in 95% of total binding of the ^{125}I labeled cytokine was defined as detection limit. The detection limit of each RIA was between 40 and 80 pg/ml.

Determination of specific binding of IL-1 to renal fibroblasts

Renal fibroblasts were grown to confluence in RPMI with 10% Serum Plus in 6 well plates (Costar). Confluent cells (approximately 1.0×10^6 fibroblasts per well) were incubated with 80,000 cpm of ^{125}I-IL-1α (specific activity 40 μCi/μg) in the presence or absence of increasing concentrations of unlabeled IL-1β or IL-1Ra. Nonspecific binding to fibroblasts was determined in the presence of 100 ng/ml of unlabeled IL-1β or IL-1Ra. Nonspecific binding was 110 $\pm$ 11 cpm and 170 $\pm$ 20 cpm on fibrosis-derived and normal renal fibroblasts, respectively.

Incubations were performed in binding buffer (RPMI containing 20 mM Hepes and 1% bovine serum albumin) for five hours at 6°C. Renal fibroblasts were then washed three times in binding buffer (4°C) and subsequently lysed in 1 ml of lysing buffer (PBS containing 1% SDS and 0.1 M NaOH). Radioactivity (counts per minute, cpm) was counted in a gamma counter. Furthermore, binding of various concentrations of ^{125}I-IL-1α to the two renal fibroblast cell lines was studied. For each concentration of ^{125}I-IL-1α, specific binding was determined by subtracting the cpm in the presence of 100 ng/ml of unlabeled IL-1Ra (nonspecific

binding) from that obtained in binding buffer alone (total binding). Specific binding curves were plotted and Scatchard plot analysis was performed. The dissociation constant (K_D = 1/-slope) and receptor sites per cell were calculated for both cell lines.

Due to low specific binding, background binding of ^{125}I-IL-1α to the polystyrene culture plates was determined. Eighty thousand cpm of ^{125}I-IL-1α were added to wells containing binding buffer but no fibroblasts. There was no detectable recovery of labeled IL-1α from the polystyrene culture plates containing no cells.

RNA isolation and gene expression for IL-1α and IL-1β

Renal and skin fibroblasts were grown to confluence in RPMI supplemented with 10% Serum Plus in 75 cm^2 culture flasks. Fibroblasts were washed in PBS and removed using rubber policemen in 10 ml of PBS. The fibroblasts were centrifuged for 10 minutes at 250 × g and cell pellets were lysed in 4 M guanidine isothiocyanate [38]. Total cellular RNA was isolated by centrifugation through 5.7 M cesium chloride in 0.1 M EDTA. The RNA pellet was resuspended in 200 μl of distilled water containing 0.1% diethyl pyrocarbonate (DEPC-H_2O) and precipitated by adding 2.5 volumes of 100% ethanol and sodium acetate (final concentrations 0.1 M). After 24 hours at −70°C, the precipitate was centrifuged in a microcentrifuge for 30 minutes at 4°C, supernatants were decanted, RNA pellets were washed in 70% ethanol, and again centrifuged for 10 minutes. Subsequently, the 70% ethanol was decanted and the pellets were dried in a speed vacuum. RNA pellets were dissolved in 100 μl of DEPC-H_2O. Total RNA was quantified by determination the ratio of UV light absorbance at 260/280 λ. Equal amounts of RNA (2.5, 1.25, 0.6, and 0.3 μg) per sample were transferred by vacuum suction to nylon membranes (Hybond-N, Amersham Corp.) using a dot blot minifold apparatus (Schleicher & Schuell, Inc., Keene, NH, USA) and fixed by short wave UV radiation. Prehybridization, hybridization and autoradiography were performed using standard techniques [38]. The IL-1β probe was a 1075-base pair fragment of the full-length cDNA clone of the IL-1β precursor [39] subcloned in pGEM2. The IL-1α probe was a 432-base pair fragment of the full length cDNA clone of the IL-1α precursor [40, 41]. The DNA was labeled with [α-^{32}P]dCTP by use of a random primer DNA labeling kit (Boehringer Mannheim, Germany). As a RNA quantity control, samples were hybridized with chicken β-actin.

Statistical analysis

Fibroblast cultures were performed in quadruplicates in 96 well plates or in duplicates in 24 well plates. For cytokine measurements, the supernatants of quadruplicate and duplicate samples were pooled. The "*N*" given in the Figure legends indicate the number of independent experiments, each representing the average of quadruplicate or duplicate samples. Results of the cytokine measurements are given as nanogram per cell number, mean ± SEM, of the number of independent experiments. Significant differences were assessed using one Factor ANOVA analysis. Post-hoc method of data evaluation was Fisher's PLSD test.

Results

IL-1 and TNF induce IL-8 and IL-6 production by renal fibroblasts

As shown in Figure 1, increasing concentrations of IL-1β and TNF-α, but not bFGF, induced IL-8 production in fibrosis-derived

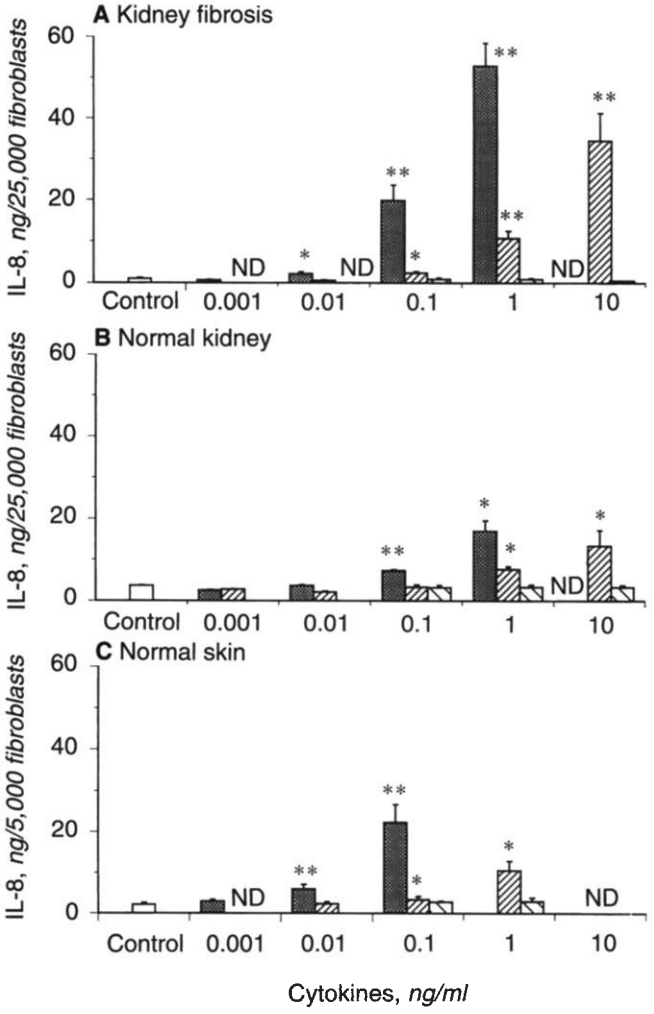

Fig. 1. *IL-8 production by fibroblasts in response to IL-1β, TNF-α, or bFGF.* Fibrosis-derived renal fibroblast (N = 8), normal renal fibroblast (N = 3) and skin fibroblast (N = 5) cultures were incubated for 24 hours in tissue culture medium containing 10% Serum Plus with and without IL-1β, TNF-α, or bFGF indicated in ng/ml under the horizontal axis. IL-8 (mean ± SEM) in supernatants is expressed in nanogram per 25,000 renal fibroblasts or in nanogram per 5,000 skin fibroblasts. *P < 0.05, **P < 0.01 compared to controls (10% Serum Plus only); n.d. = not done.

renal fibroblasts. A similar induction was observed in fibroblasts derived from healthy kidney and skin. At 10 to 100 pg/ml (600 fM to 6 pM), IL-1β induced significant amounts of IL-8 (P < 0.05 and P < 0.01, respectively). On a molar basis, IL-1β was approximately 10-fold more potent than TNF-α in inducing IL-8 in each of the three fibroblast cell lines. Fibrosis-derived renal fibroblasts produced three- to fourfold more IL-8 than normal renal fibroblasts. The differences in cytokine-induced IL-8 production per 25,000 renal fibroblasts was significant (P < 0.05) in response to IL-1β (0.1 and 1 ng/ml) and high dose TNF-α (10 ng/ml).

The pattern of IL-1β and TNF-α-induced IL-6 production by fibroblasts (Fig. 2) is similar to that for IL-8. Basic FGF does not induce IL-6 above control levels, and IL-1β is approximately 10 times more potent than TNF-α in inducing IL-6 in each of the fibroblast cultures. Compared to normal renal fibroblasts, fibrosis-derived renal fibroblasts tended to produce more IL-6 per 25,000 cells.

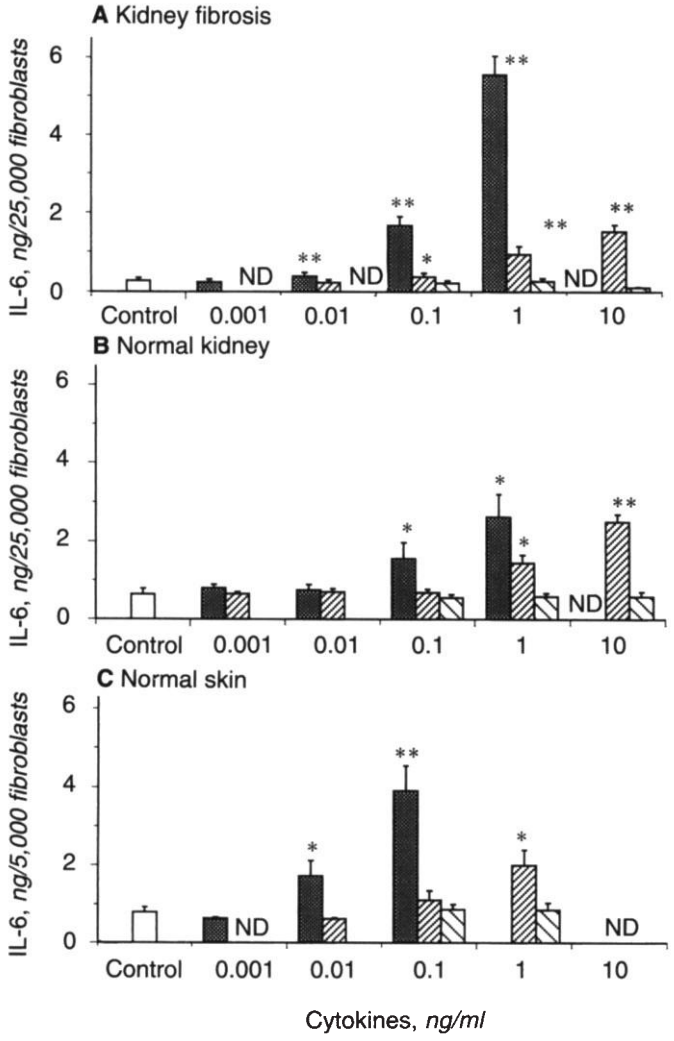

Fig. 2. *IL-6 production by fibroblasts in response to IL-1β, TNF-α, or bFGF.* Under the conditions described in Figure 1, fibrosis-derived (N = 8) as well as normal renal fibroblast (N = 3) and skin fibroblast (N = 5) cultures were stimulated for 24 hours with IL-1β, TNF-α, or bFGF. Cytokine-induced IL-6 in fibroblast supernatants is depicted in nanogram per 25,000 renal fibroblasts or in nanogram per 5,000 skin fibroblasts. The mean ± SEM are shown. $^*P < 0.05$, $^{**}P < 0.01$ compared to controls; n.d. = not done.

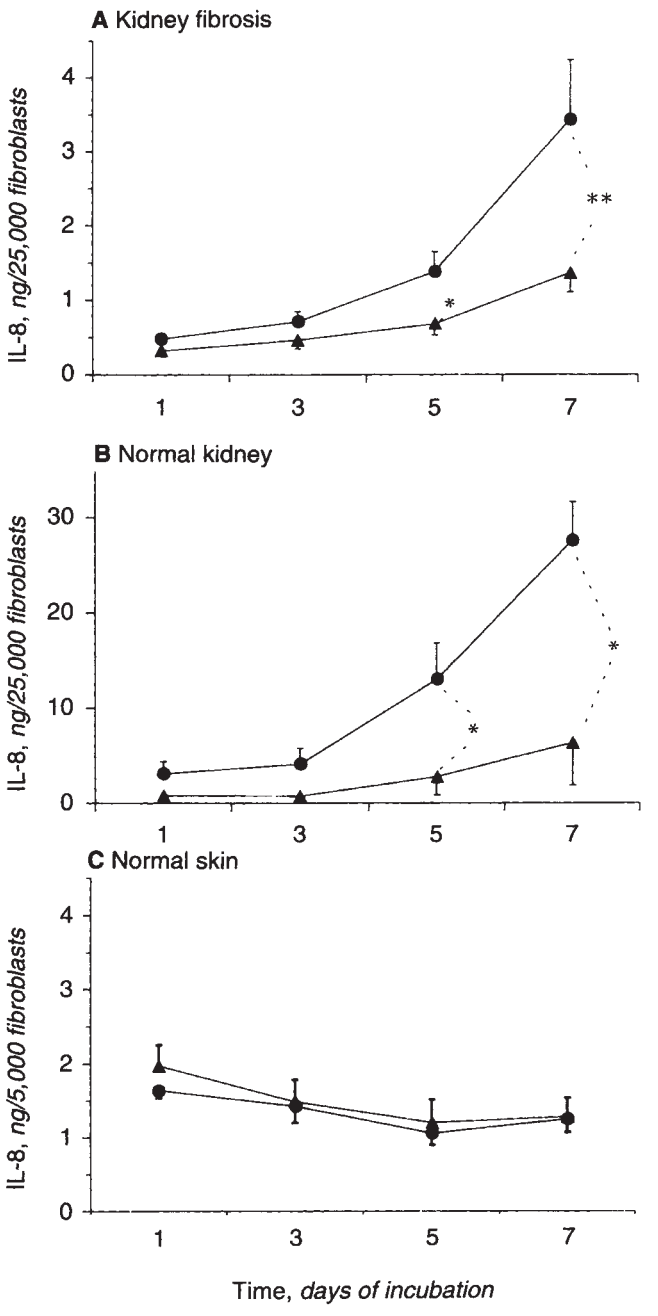

Fig. 3. *Effect of IL-1Ra on spontaneous IL-8 production from fibroblasts.* Fibrosis-derived renal fibroblast (N = 6), normal renal fibroblast (N = 3), or skin fibroblast (N = 4) cultures were incubated for 1, 3, 5, or 7 days in media containing 10% Serum Plus without (closed circles) and with IL-1Ra at 1 μg/ml (closed triangles). IL-8 production in nanograms is expressed per 25,000 renal fibroblasts and 5,000 skin fibroblasts, respectively. Symbols represent the mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$ comparing controls (10% serum) to samples with IL-1Ra at the same time.

Spontaneous IL-8 and IL-6 production by renal fibroblasts is IL-1 dependent

In a second set of experiments, renal and skin fibroblasts were incubated for various periods of time under conditions of IL-1 receptor blockade. To accomplish this, after trypsinization, each fibroblast suspension was split into two aliquots, one containing tissue culture medium alone (control) and the second containing 1 μg/ml IL-1Ra. As shown in Figures 3 and 4, fibrosis-derived as well as normal renal fibroblasts spontaneously produce IL-8 and IL-6 as detected in the fibroblast supernatants after 1, 3, 5, and 7 days of incubation. To compensate for the growth of cells during these time periods, cytokine concentrations were corrected for 25,000 renal fibroblasts and 5,000 skin fibroblasts, respectively (**Methods**). IL-8 and IL-6 production per 25,000 fibrosis-derived renal fibroblasts or normal renal fibroblasts increased over time. However, this spontaneous IL-8 and IL-6 production was inhibited in cultures containing IL-1Ra. In contrast, spontaneous production of IL-8 and IL-6 by skin fibroblasts did not increase over time and was not affected by IL-1Ra. Similar results were observed when fibroblasts were grown in tissue culture medium supplemented with 1% Serum Plus (data not shown).

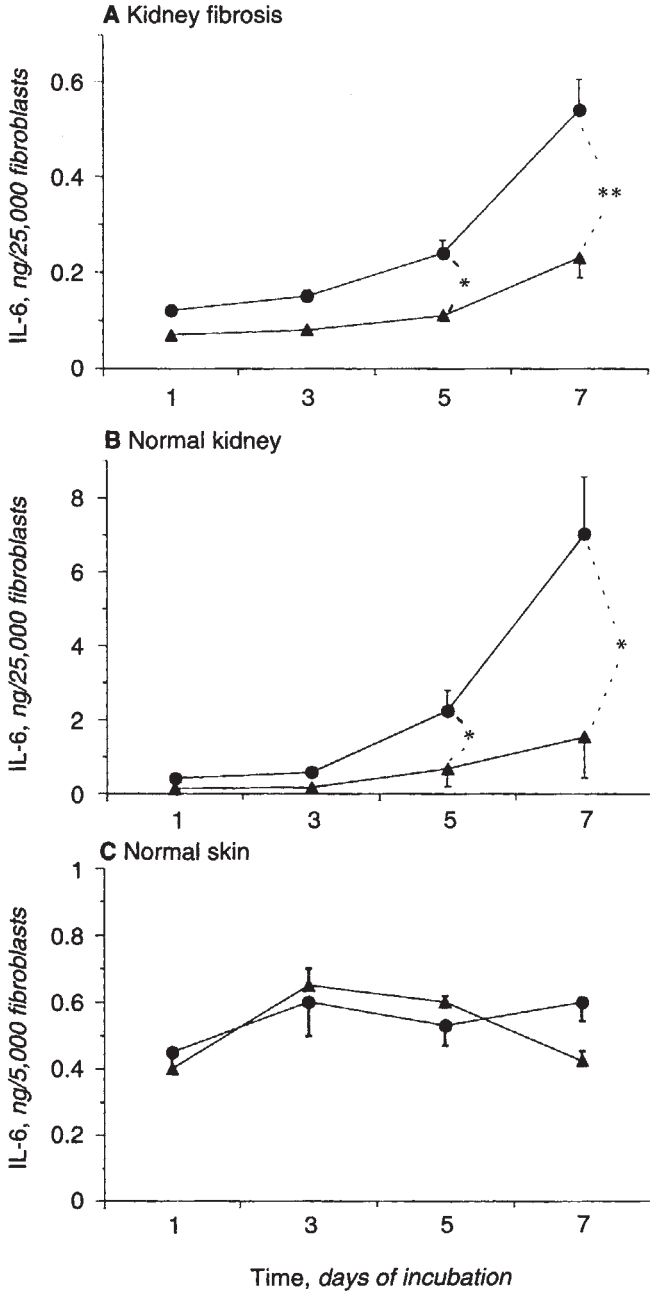

Fig. 4. *Effect of IL-1Ra on spontaneous IL-6 production from fibroblasts.* Fibrosis-derived ($N = 6$), normal renal fibroblast ($N = 3$) and skin fibroblast ($N = 4$) cultures were incubated for 1, 3, 5, or 7 days under the conditions described in Figure 3. Closed circles represent cultures in 10% Serum Plus only; closed triangles depict 10% Serum Plus + IL-1Ra at 1 μg/ml; mean ± SEM; $^*P < 0.05$, $^{**}P < 0.01$ comparing 10% serum to 10% serum plus IL-1Ra at the same time.

Dose response of IL-1Ra-mediated inhibition of renal fibroblast IL-8 and IL-6 production

Fibroblasts were incubated for four days with or without IL-1Ra (Fig. 5 A, B). The effect of IL-1Ra on spontaneous IL-8 and IL-6 production by renal fibroblasts was dose-dependent, reached significance at 0.1 to 1 ng/ml ($P < 0.05$), and was maximal at 10 μg/ml. At this concentration, IL-1Ra inhibition was 37 to 57% in fibrosis-derived as well as normal renal fibroblasts. There was no significant effect of IL-1Ra on skin fibroblasts at any concentration of IL-1Ra.

Gene expression and protein synthesis of IL-1β and IL-1α by renal fibroblasts

Fibrosis-derived and normal renal fibroblasts spontaneously expressed mRNA coding for IL-1β and IL-1α (Fig. 6). Total RNA was isolated from confluent fibroblast monolayers. For both renal cell lines, this occurred after seven days and for skin fibroblasts after 14 days of culture in 10% serum. As demonstrated by dot blot analysis, normal and fibrosis-derived renal fibroblasts have similar steady state levels of IL-1 mRNA. For comparison, 5 μg of total RNA were extracted from skin fibroblasts. Skin fibroblasts expressed similar levels of mRNA coding for IL-1α but less IL-1β mRNA than both kidney fibroblasts cell lines.

We also measured synthesis of IL-1α and IL-1β in renal fibroblasts. As IL-1 concentrations in the culture supernatants were at or below the detection limit of the RIAs (40 to 80 pg/ml), fibroblasts were lysed by three freeze-thaw cycles and total IL-1 production (cell-associated plus secreted) was determined. There was no detectable IL-1α or IL-1β protein in skin fibroblasts (data not shown). In contrast, both IL-1 peptides were detectable in renal cells, and spontaneous production of IL-1α was twice as high as that of IL-1β ($P < 0.05$) in both renal cell lines. Furthermore, total IL-1 synthesis was greater in normal renal fibroblasts ($P < 0.05$ for IL-1β; $P < 0.1$ for IL-1α) compared to fibrosis-derived renal fibroblasts (Fig. 7). These data correspond to the higher spontaneous, IL-1-dependent IL-6 and IL-8 production by normal renal fibroblasts compared to fibrosis-derived cells (Figs. 3 and 4).

IL-1 induces IL-1 in renal fibroblasts

Normal as well as fibrosis-derived renal fibroblasts were grown to confluence in 24-well culture plates and subsequently incubated for three days in serum-free media in the presence and absence of increasing amounts of IL-1β. Fibroblasts were lysed by three freeze-thaw cycles and total (cell-associated plus secreted) IL-1α was measured by RIA. As shown in Figure 8, IL-1β-induced IL-1α increased dose-dependently. At 1 ng/ml IL-1β, IL-1α production increased from 76 ± 7 pg to 183 ± 31 pg per 200,000 cells ($P < 0.05$) in fibrosis-derived renal fibroblasts and from 144 ± 13 pg to 235 ± 14 pg per 200,000 cells ($P < 0.01$) in normal renal fibroblasts. IL-1α production by both renal cell lines increased further in response to 10 and 100 ng/ml of IL-1β.

Determination of specific IL-1 binding sites on renal fibroblasts

The specific binding of ^{125}I-IL-1α was determined in the two renal cell lines. Each renal fibroblast cell line specifically bound approximately 0.1% of the total label as demonstrated by the binding curves (Fig. 9A) and Scatchard plot analysis (Fig. 9B). Based on these data, the calculated dissociation constant (K_D) for normal renal fibroblasts and fibrosis-derived renal fibroblasts were 0.53 pM and 0.66 pM, respectively. The calculated number of receptor sites per cell were 1,080 per normal renal fibroblast and 600 receptors per fibrosis-derived renal fibroblast. When taken together, these data demonstrate that both renal fibroblast cell lines express relatively few IL-1 receptor sites per cell with high affinity. As shown in Figure 10 A and B, the binding of IL-1α to fibrosis-derived or normal renal fibroblasts was inhibited by either IL-1Ra or IL-1β. Inhibition of binding by IL-1Ra was similar to that by IL-1β.

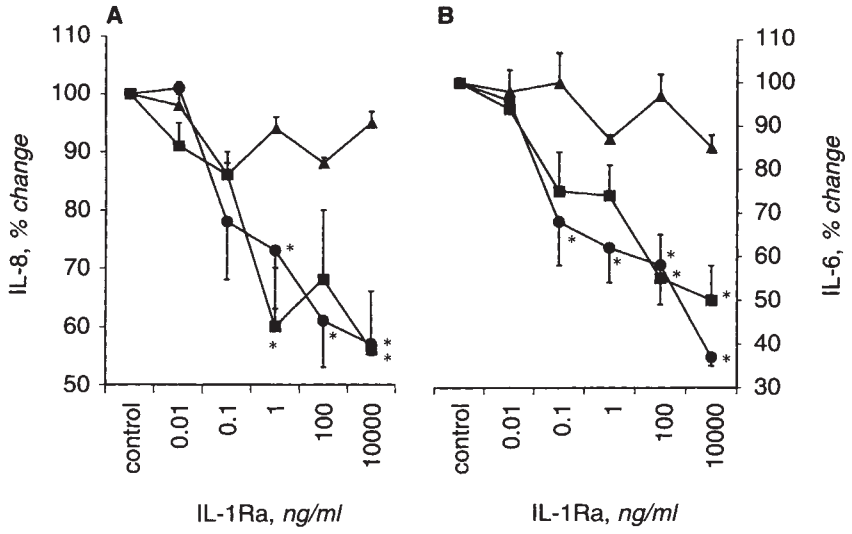

Fig. 5. *Dose-response of IL-1Ra on spontaneous production of IL-8 and IL-6.* **A.** IL-8; **B.** IL-6. Fibrosis-derived renal fibroblasts (closed circles), normal renal fibroblasts (closed squares), and skin fibroblasts (closed triangles) were incubated for 5 days in media containing 10% Serum Plus in the absence or presence of increasing concentrations of IL-1Ra. IL-8 and IL-6 production in the presence of IL-1Ra are expressed as the per cent of that from control (cells in 10% Serum Plus only). Data represent the mean ± SEM of 3 separate experiments.

Discussion

Human renal interstitial fibroblasts are likely to participate in the pathogenesis of interstitial inflammation with fibrosis due to proliferation and matrix production [23]. The present study provides evidence that renal fibroblasts are also a source of the chemokine IL-8 as well as the growth-promoting cytokine IL-6. Cultured renal fibroblasts spontaneously produce IL-8 and IL-6 and approximately 50% of this spontaneous production is dependent on the intrinsic synthesis of IL-1. The spontaneous production of IL-6 and IL-8 is higher in renal fibroblasts derived from a normal kidney than in those obtained from a kidney with glomerulonephritis and interstitial fibrosis. This increased synthesis is associated with an increased spontaneous production of IL-1α and IL-1β. In addition, IL-1β induced more IL-1α in normal than in fibrosis-derived renal fibroblasts. There is also a higher expression of specific IL-1 binding sites on normal renal fibroblasts compared to fibrosis-derived renal fibroblasts. As both renal fibroblast cell lines similarly express the genes for IL-1α and β, these data suggest that post-transcriptional events involving translation and processing of IL-1 as well as expression of IL-1 receptors are downregulated in unstimulated fibrosis-derived fibroblasts compared to normal renal cells.

Although mRNA coding for IL-1α and β in primary skin fibroblasts was observed, IL-1 synthesis, however, was not detected in these cells. These data are in agreement with a study by Elias et al who described accumulation of IL-1β mRNA in IL-1 (α or β) stimulated fibroblasts without translation into the IL-1 precursor [42]. The authors concluded that IL-1 production in human lung fibroblasts is inhibited on a post-transcriptional level [42]. According to our data, a similar post-transcriptional regulation seems to occur in skin fibroblasts. In contrast, post-transcriptional blockade of IL-1 synthesis is not complete in the two renal fibroblast cell lines tested in this study. IL-1-producing fibroblasts may be due to organ specificity or secondary to the SV40 transformation.

The number of specific IL-1 binding sites on the two transformed fibroblast cell lines of kidney origin was relatively small with 600 to 1080 sites per cell. Of the two IL-1 receptors, the type I IL-1 receptor accounts for IL-1 binding to renal fibroblasts because of its high affinity for IL-1α as indicated by the K_D of approximately 1×10^{-12} M. This high affinity is comparable to that of IL-1 receptors determined on human lung fibroblasts [43], or normal human skin fibroblasts [44]. The number of binding sites per cell, however, was higher with 1,600 per cell on skin fibroblasts [44] and 3,000 per cell on lung fibroblasts [43].

Explanations for the expression of low number of receptors on fibrosis-derived renal fibroblasts remain speculative. However, it is possible that there are reduced numbers of unoccupied receptors because these cells spontaneously produce IL-1. It has been shown that receptor occupancy of less than 5% by IL-1 is able to downregulate the surface expression of the IL-1 receptor type I [45]. Therefore, the reduced expression of IL-1 receptors on fibrosis-derived renal fibroblasts may reflect chronic stimulation of these cells by IL-1 itself.

The production of IL-6 and IL-8 in response to picogram concentrations of exogenous IL-1β was higher in fibrosis-derived than in normal renal fibroblasts. According to our data, this enhanced responsiveness is neither explained by differences in the number of IL-1 binding sites nor by a change in the affinity of the IL-1 receptor on fibrosis-derived fibroblasts. We speculate that signal transduction pathways for IL-1-induced IL-6 and IL-8 production are more efficient in fibrosis-derived renal fibroblasts than in cells derived from normal kidneys.

Compared to primary skin fibroblasts, the pattern of IL-8 and IL-6 production in response to exogenous IL-1β and TNF-α was similar in the two renal fibroblast cell lines. On a molar bases, IL-1β was approximately 10-fold more potent than TNF-α in inducing IL-6 and IL-8 in each cell line. These results are in agreement with previous studies using rat kidney fibroblasts [46] and fibroblasts derived from human skin [47, 48], synovial tissue [49, 50] or lung [51–53]. Recent data further suggest that IL-1 and TNF-α may act synergistically in inducing IL-8 production in fibroblasts [54]. In contrast, bFGF did not induce IL-6 or IL-8 above background in the three cell lines and did not enhance IL-1 or TNF-α-induced IL-8 or IL-6 production (data not shown). These data support the concept that IL-8 as well as IL-6 production by fibroblasts, including renal fibroblasts, depends on the stimulation by pro-inflammatory cytokines such as IL-1 and TNF-α rather than on growth factors.

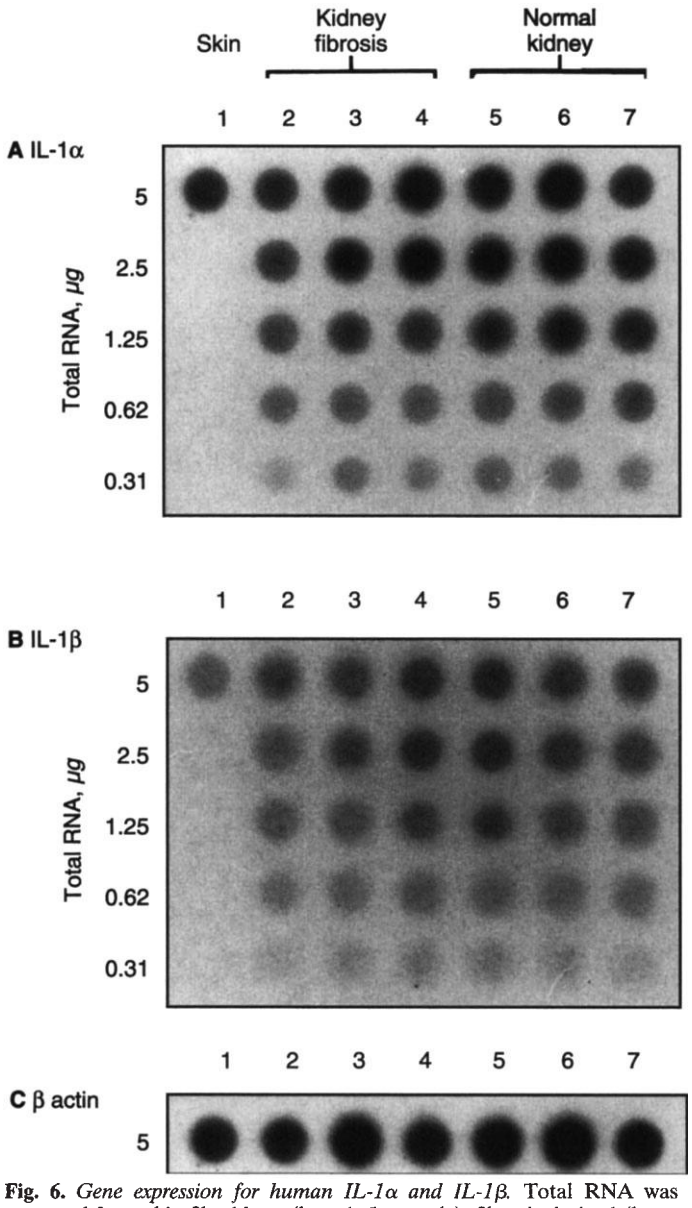

Fig. 6. *Gene expression for human IL-1α and IL-1β.* Total RNA was extracted from skin fibroblasts (lane 1, 5 μg only), fibrosis-derived (lanes 2 to 4) as well as normal renal fibroblasts (lanes 5 to 7) after reaching confluence (after 7 and 14 days for renal and skin cells, respectively). RNA for renal fibroblasts were diluted (5, 2.5, 1.25, 0.62, and 0.31 μg/well) and the membrane hybridized with cDNA probes coding for human IL-1α, human IL-1β, and β-actin as an RNA control.

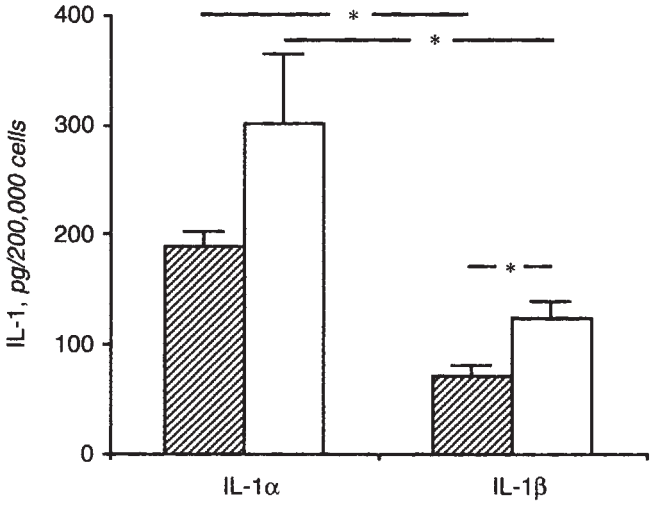

Fig. 7. *Spontaneous IL-1α and IL-1β production from fibroblasts.* Fibrosis-derived renal fibroblast (hatched bars) and normal renal fibroblast (open bars) cultures were incubation for 5 days in media containing 1% Serum Plus. Cells were lysed by three freeze-thaw cycles and total production (cell-associated plus secreted) of IL-1α and IL-1β was measured by specific RIA. IL-1 production is expressed in picogram per 200,000 fibroblasts. The bars represent the mean ± SEM of 4 independent experiments done in duplicates; $^*P < 0.05$ as indicated by horizontal lines. The difference in IL-1α production was not significant ($P < 0.1$).

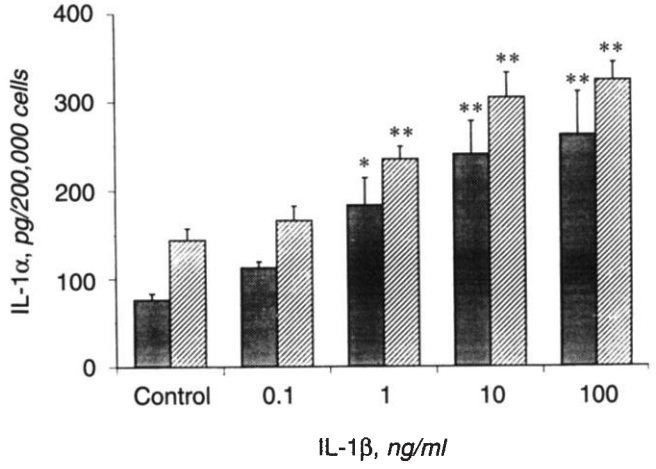

Fig. 8. *IL-1 induces IL-1 in renal fibroblasts.* Fibroblasts derived from kidney fibrosis and normal kidney were grown to confluence in 24 well plates and incubated for 3 days in serum-free media in the absence and presence of the indicated amounts of IL-1β. Total IL-1α production is expressed in picogram per 200,000 fibroblasts. The bars represent the mean ± SEM of 4 experiments; $^*P < 0.05$, $^{**}P < 0.01$ compared to control (no IL-1).

In the preceeding paper, we described the spontaneous proliferation of fibrosis-derived renal fibroblasts but not that of normal renal fibroblasts was IL-1-dependent because IL-1 receptor blockade using IL-1Ra reduced growth. We concluded from those results that renal fibroblasts derived from diseased kidneys are different from normal renal fibroblasts in that the former produce IL-1 as a paracrine growth factor. We now demonstrated that both kidney fibroblast cell lines spontaneously produce IL-1α and IL-1β, and that this endogenous IL-1 induces IL-6 production via an IL-1 paracrine loop. The fact that the spontaneous proliferation of normal renal fibroblasts was not inhibited by IL-1Ra, although these cells produce IL-1, suggests that signal transduction pathways for IL-1-induced IL-6 are different and independently regulated from those of IL-1-induced proliferation [55, 56].

Infiltrating leukocytes including granulocytes, monocyte/macrophages and lymphocytes play a critical role in several models of renal disease [57, 58]. Leukocytes are attracted by the chemokines including IL-8 and MCP-1 which are produced by intrinsic cells at the site of inflammation [59]. Although the chemotactic activity of IL-8 was originally described for neutrophils [60, 61], low concentrations of IL-8 are chemoattractant for lymphocytes *in vivo* [62].

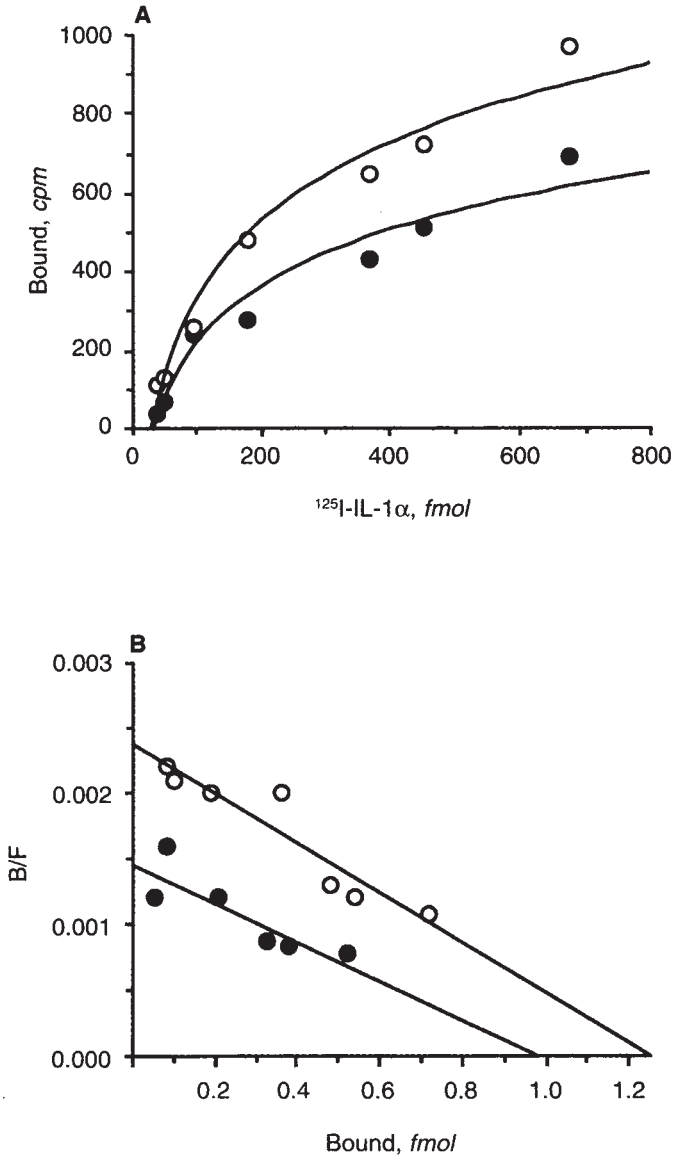

Fig. 9. *Specific binding of IL-1 to renal fibroblasts.* Renal fibroblasts were incubated for 5 hours at 6°C in binding buffer containing increasing amounts of ^{125}I-IL-1α in the presence (nonspecific binding) or absence (total binding) of 100 ng/ml IL-1Ra. Specific binding (total-nonspecific) is shown in **A**. The Scatchard plot (**B**) shows reduced binding sites on fibrosis-derived compared to normal renal fibroblasts. The K_D was similar with 0.53 pmol for normal renal fibroblasts and 0.66 pmol for fibrosis-derived renal fibroblasts. The mean of duplicate data of 1 out of 3 similar experiments are shown.

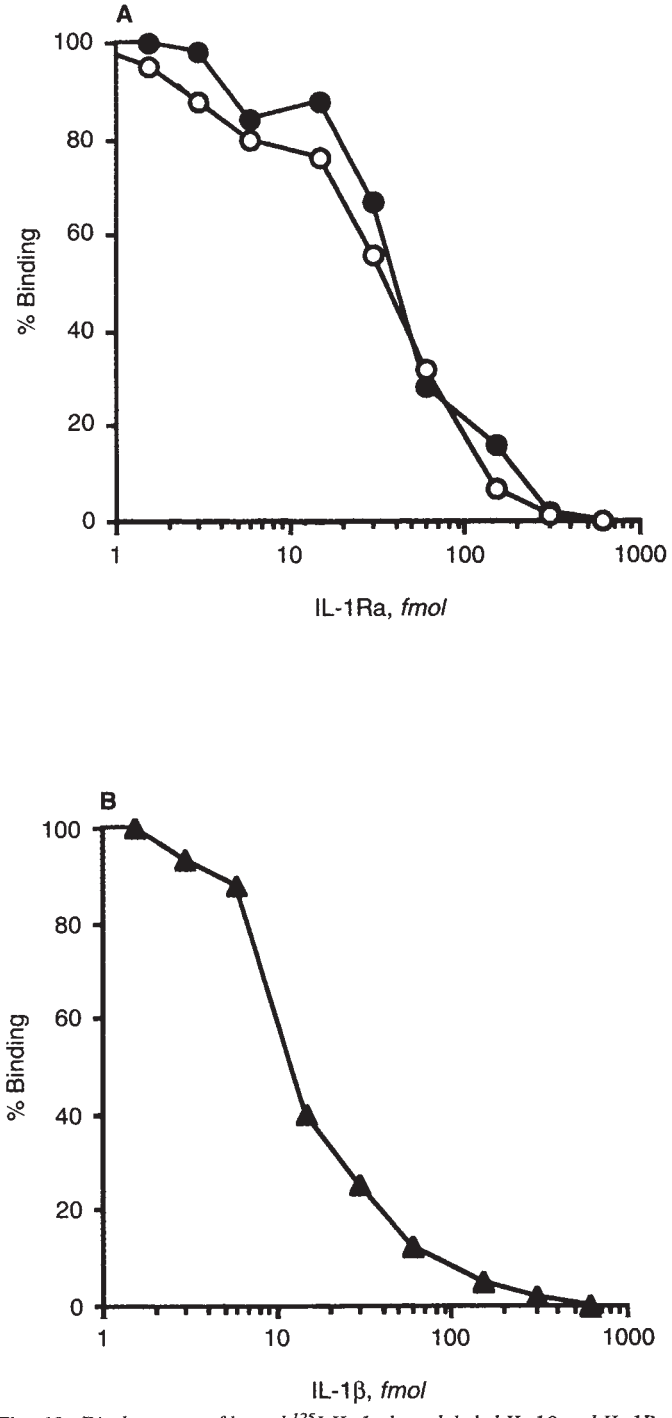

Fig. 10. *Displacement of bound ^{125}I-IL-1α by unlabeled IL-1β and IL-1Ra.* Renal fibroblasts were incubated for 5 hours at 6°C in binding buffer containing 80,000 cpm of ^{125}I-IL-1α per 1×10^6 cells in the presence or absence of increasing concentrations of unlabeled IL-1Ra (**A**) or unlabeled IL-1β (**B**).

This may be important with respect to the renal interstitial infiltrate which contains predominantly lymphocytes and macrophages and very few granulocytes [21, 22].

Cultured human glomerular mesangial cells produce IL-8 [8] and MCP-1 [11], and it has been demonstrated that the release of these chemotactic factors from mesangial cells was inhibited by IL-1Ra [63, 64]. In the present paper we demonstrate that a similar mechanism is present in renal interstitial fibroblasts. These data suggest that in renal inflammation, renal cells including mesangial cells and interstitial fibroblasts release chemoattractant activity with the consequence of leukocyte infiltration which depends on the intrinsic production of IL-1.

Like spontaneous IL-8 production, IL-6 is inhibited by IL-1Ra in mesangial cells [63], and in the present study we observed this dependency in renal fibroblasts. Although IL-6 has been initially described to enhance proliferation of mesangial cells in culture

[14], the growth-promoting activity of IL-6 is controversial in that in studies exogenous IL-6 has an inhibitory effect on mesangial cell proliferation [65]. Furthermore, using a mouse model of IgA nephropathy, Montinaro et al showed that administration of IL-6 in combination with IL-1, but not IL-6 alone, induced hypercellularity in the glomerulus [66]. Although both renal fibroblast cell lines produce IL-6, it remains to be elucidated whether these cells use IL-6 as a growth factor.

In summary, human renal interstitial fibroblasts in culture spontaneously produce IL-6 and IL-8. This spontaneous synthesis of growth promoting as well as chemotactic activity depends, in part, on the intrinsic production of IL-1. The paracrine IL-1 loop promotes spontaneous proliferation as well as IL-8 and IL-6 production in fibrosis-derived renal fibroblasts. Therefore, IL-1 synthesis by interstitial fibroblasts is likely to contribute to the development of interstitial fibrosis in the course of chronic inflammatory kidney diseases. It remains to be established whether a down-regulation of renal-fibroblast IL-1 production, for example by IL-1Ra, could be beneficial to prevent interstitial inflammation with subsequent fibrosis.

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